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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of	)	
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Klein et al.	)	Group Art Unit: 1632
	)	
Serial No. 09/374,742	)	Examiner: Baker, A.
	)	
Filed: August 13, 1999	)	
	)	
For: "METHODS AND COMPOSITIONS FOR BISUBSTRATE INHIBITORS OF ACETYLTRANSFERASES"	)	

DECLARATION OF DR. DAVID KLEIN UNDER 37 C.F.R. § 1.132

BOX AF
Assistant Commissioner of Patents
Washington, DC 20231

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I, David Klein, a citizen of the United States of America, residing at 6112 Tilden Lane, Rockville, MD 20852, USA, declare that:

1. I am a co-inventor of the above-referenced patent application and of the subject matter described and claimed therein.

2. I have a Ph.D. degree in biology from Rice University in Houston, Texas. I have been conducting research in the field of endocrinology since 1965 and am a co-author of at least 300 publications relating to endocrinology in general, the majority of which deal with arylalkylamine N-acetyltransferase and melatonin production. I am currently Chief of the Section on

Neuroendocrinology, Laboratory of Developmental Biology at the National Institute of Child Health of the National Institutes of Health in Bethesda, Maryland.

3. Studies conducted in my laboratory demonstrate that additional alkylating derivatives of an acetyl acceptor substrate for an acetyltransferase and additional acetyltransferases can be used to produce the bisubstrate inhibitor described in the present invention.

4. Specifically, presented herein are data which demonstrate the inhibition of enzyme activity as a result of bisubstrate inhibitor production with bromoacetylkanamycin as the alkylating derivative of the acetyl acceptor substrate and kanamycin-6' -N-acetyltransferase as the acetyltransferase. Both bromoacetylkanamycin and kanamycin 6'-N-acetyltransferase are disclosed in Table 1 of the present application. Kanamycin 6'-N-acetyltransferase is a different acetyltransferase than AANAT and bromoacetylkanamycin, which is specific for kanamycin 6'-N-acetyltransferase, is a different alkylating derivative of an acetyl acceptor substrate than N-bromoacetyltryptamine (BAT) and N-chloroacetyltryptamine, both of which are specific for AANAT.

5. The experiments which provided the data described above were conducted as described herein and as set forth in the Examples in the present application. Specifically, bromoacetylkanamycin was synthesized by SynPeP Corporation, Dublin, CA. The preparation contained a mixture of mono- and di- bromoacetylated kanamycin. Kanamycin 6'-N-acetyltransferase was isolated from *E. coli* and was provided as a lyophilized powder (Sigma Chemicals). The enzyme assay was done essentially as described (Benveniste and Davies, "Enzymatic acetylation of aminoglycoside antibiotics by *E. coli* carrying an R factor." Biochemistry 10:1787-1796 (1971)). Increasing concentrations (1 μM to 1 mM) of the bromoacetylkanamycin preparations were incubated (30°C, 20 min) with the enzyme in the presence of kanamycin (0.5 mM) and ^{3}H-acetyl-CoA (0.5 mM, 4Ci/mole) in a total volume of 50

µl Tris HCl (100 mM, pH = 6.7). Following the incubation, 25 µl aliquots of the preparation were spotted on phosphocellulose paper (Whatman P-81) and then immersed in hot distilled water (70-80°C) for 2 min to stop the reaction. The paper was then washed (5 x 10 minutes) with large volumes of distilled water. Individual spots were cut out and treated with 0.1 N NaOH (1.0 ml) to elute the bound ^{3}H-acetylkanamycin and the radioactivity in 0.5 ml aliquots was determined.

6. The data produced from these studies demonstrated that the preparation containing mono- and di- bromoacetylated kanamycin effectively inhibits kanamycin 6'-N-acetyltransferase activity (See Figure 1, attached hereto as Exhibit 1). The IC_{50} value represents a concentration of the bromoacetylkanamycin preparation that inhibits the enzyme activity by 50%. The IC_{50} value (19 mM) for the preparation containing mono- and di- bromoacetylkanamycin is an overestimate since this preparation contained multiple species with different inhibitory effect. In the case of serotonin N-acetyltransferase, it has been observed that the inhibitory potency parallels substrate specificity. Therefore, it is reasonable that 6'-bromoacetylkanamycin has the highest potency in the present studies. The relative concentration of 6'-bromoacetylkanamycin in the present preparation is not known. Therefore, the IC_{50} value was not determined accurately.

7. Furthermore, studies conducted in my laboratory have demonstrated that BAT analogs, i.e. different alkylating derivatives, function to produce a bisubstrate inhibitor in a cell.

8. The potency of BAT as an inhibitor of AANAT is a function of the aromatic group by using analogs in which the aromatic groups were different. The effectiveness of the analogs was determined by incubating the compounds with human AANAT. The analogs were dissolved in ethanol and subsequently diluted in sodium phosphate buffer (0.1 M, pH 6.8) containing 2% ethanol to make a stock solution that was 4X, relative to the highest concentration used. This was then diluted as required.

9. A range of concentrations of the compounds was tested by incubating the compounds (37° C, 15 min) with purified enzyme preparation in the presence of [^{3}H]-acetyl CoA (4 Ci/mol. 0.5 mM) and tryptamine (1 mM) in a total volume of 0.1 ml. Following incubation, the [^{3}H] acetyltryptamine was extracted using chloroform, washed twice using 1 N NaOH (0.2 ml), and radioactivity in 0.4 ml of chloroform was determined. This experiment was done with human AANAT (hAANAT) (results shown in Table 2 and Figure 1 in Exhibit 3) and ovine AANAT (data not presented). The identifying numbers (I.D.#) of the analogs and their chemical structures are S 27479-1 (2-Bromo-N-[2-(5-methoxy benzothiophen-3-yl) ethyl] acetamide); S 27535-1 (2-Bromo-N-[2-(5-fluoro benzothiophen-3-yl) ethyl] acetamide); S 27244-1 (2-Bromo-N-[2-(7-hydroxy napht-1-yl) ethyl] acetamide); S 20251-1 (2-Bromo-N-[2-(7-methoxy napht-1-yl) ethyl] acetamide). BAT was tested as a control and therefore, it was possible to estimate the IC50 values and potency relative to BAT (Table 2, Exhibit 3).

10. These results teach that the potency of BAT as an inhibitor of AANAT is a function of the aromatic ring and demonstrate that changing this ring will influence potency. It is also clear from this study that a more potent analog S 27244-1 has the structure of 2-bromo-N-[2-(7-hydroxy napht-1-yl) ethyl] acetamide and teaches that substitution of the indole ring of BAT with the 7-hydroxynaphtyl ring increases potency.

11. The results of the studies described above demonstrate that additional acetyl acceptor substrates and additional acetyltransferases can be employed in the claimed methods of this invention to produce a bisubstrate inhibitor by a common mechanism.

12. Furthermore, studies conducted in my laboratory have demonstrated that a method of producing a bisubstrate inhibitor in a cell which is *in vivo*.

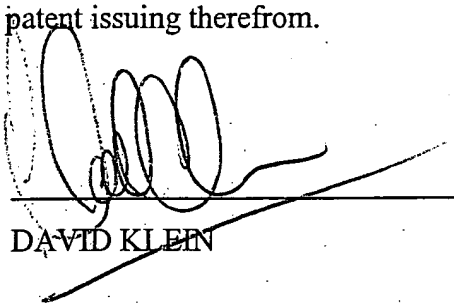
13. Specifically, presented herein are data which demonstrate that the administration of bromoacetyltryptamine to rats results in the inhibition of melatonin production by pinealocytes. The production of melatonin in pinealocytes is directly controlled by the acetyltransferase, AANAT. Bromoacetyltryptamine(BAT) has been identified as a potential inhibitor of melatonin synthesis, as shown by its inhibitory effects on AANAT in pinealocytes in cell culture. The rats used in these experiments were treated to elevate melatonin production. BAT was administered to these animals and assays for pineal melatonin production in these animals were conducted one hour after BAT administration. The measurement of pineal melatonin production is an established index of the rate of ongoing melatonin synthesis.

14. The experiments which provided the data described above were conducted as described herein and as set forth in the Examples in the present application. Specifically, rats (24 days old, ~60 grams) were treated with isoproterenol (1 mg/kg, sc) to elevate melatonin production. Following a 0.5 hour period, animals were treated with BAT (~60 mg/kg) and then were killed one hour later. Pineal glands were removed, homogenized and melatonin was measured by radioimmunoassay, according to assays known in the art and as described in the present application.

15. Data generated from these experiments demonstrated that treatment with BAT did not alter the general health of the animal, as indicated by visual examination. Furthermore, changes in breathing rate, balance or activity were not detected. However, BAT treatment was shown to result in significantly lower levels of pineal melatonin in these animals (See Table 1, attached hereto as Exhibit B).

16. These studies demonstrate that BAT inhibits melatonin synthesis *in vivo* by producing a bisubstrate inhibitor upon contact with its target enzyme, AANAT, thereby demonstrating that the methods of the present invention can be carried out in a cell *in vivo*.

17. I further declare that all statements made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under § 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or document or any patent issuing therefrom.



DAVID KLEIN

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